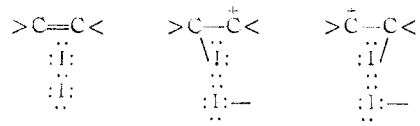
the error in estimating ϵ_c values from the ordinate intercepts in the plot of equation (3), *cf.* Fig. 2, is large.

It is interesting to note that the K value for the iodine–cyclohexene complex is higher than that measured by Benesi and Hildebrand for the iodine benzene complex in *n*-heptane (1.18), and the maximum extinction coefficients of the cyclohexene and benzene complexes are of the same order of magnitude.

These iodine complexes at the double bond may be structurally similar to the cyclic halonium ion so often discussed as an intermediate in the addition of halogen to the double bond. Certainly a structure of the type, as represented by the resonance forms



is in keeping with the accepted structures for the olefin–silver ion complexes.[2a] It seems likely that the characteristic absorption peaks of these complexes may be interpreted in terms of an intermolecular charge-transfer process, as has been done previously for the aromatic–halogen complexes.[4]

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(4) R. S. Mulliken, THIS JOURNAL, **72**, 605 (1950); **74**, Feb. (1952).

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Experimental Investigation of Fractionation in the Electrophoresis–Convection Apparatus

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A study has been made of the fractionation by electrophoresis–convection of systems containing two protein components. The experimental separation factors are in excellent agreement with the theoretical separation factors and as in the case of single protein systems the experimental rate of transport is in moderately good agreement with the theory. The experimental times of transport correspond to effective field strengths bearing ratios of between unity and one-fifth to the nominal applied field strength.

Introduction

In view of the increasing importance of electrophoresis–convection as a simple convenient method of fractionating aqueous solutions of protein mixtures a systematic experimental study of the conditions determining the rate of transport and the degree of fractionation of proteins by electrophoresis–convection has been undertaken. The results of the investigation of transport in systems containing a single mobile protein component have been presented in a previous article.[2] The results of a study of the fractionation by electrophoresis–convection of systems containing two non-isoelectric protein components is reported in the present paper.

The electrophoresis–convection apparatus as described by Cann, Kirkwood, Brown and Plescia[3] consists of two reservoirs connected by a narrow, vertical, semipermeable channel formed between two sheets of Visking Corporation sausage casing. The top of the upper reservoir is open so that the reservoirs and channel may be filled with a solution of the proteins to be fractionated. In operation the apparatus is filled and immersed in a suitable buffer solution between two flat platinum electrodes arranged to provide a homogeneous electric field across the channel upon the passage of a direct current between them. The field effects a horizontal transport of the protein components establishing horizontal density gradients in the channel, which, under the action of gravity, result in differential migration of the proteins into the lower reservoir. In the case of a single protein system the protein is simply concentrated in the bottom reservoir. External circulation of the buffer is maintained to prevent accumulation of electrolysis products.

The theory of electrophoresis–convection developed by Kirkwood and co-workers[4] predicts that for the general multi-component system $(C_i/C_i^\circ)^{\mu j} = (C_j/C_j^\circ)^{\mu i}$ at the time of sampling for any two components i and j of the system, where C_i/C_i° is the ratio of the concentration of component i in the top reservoir to its initial concentration and μ_i is the electrophoretic mobility of component i. From this relation the separation factor, f_2, in the top reservoir for the general two component system can be shown to satisfy

$$(1 + X_2^\circ(f_2 - 1))^\beta / f_2 = \gamma^\beta$$
$$f_2 = (C_2/C_2^\circ)/(C_1/C_1^\circ) \qquad (1)$$
$$\beta = 1 - \mu_2/\mu_1$$

where $\gamma = (C_1 + C_2)/(C_1^\circ + C_2^\circ)$ is the weight fraction of total protein remaining in the top reservoir, and $X_2^\circ = C_2^\circ/(C_1^\circ + C_2^\circ)$ is the initial weight fraction of component 2. Table I presents f_2 as a function of X_2°, β and γ, as calculated from Eq. (1).

The time required to transport a specified fraction of protein from the top to the bottom reservoir of the apparatus can be calculated from the theory. However, in the case of two components, the integrals involved depend upon too many parameters characteristic of the particular pair of proteins to make a systematic tabulation practical.

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TABLE I

SEPARATION FACTORS, f_2, IN TOP RESERVOIR FOR THE TWO COMPONENT SYSTEM

γ = residual fraction of total protein in top; X_2° = initial weight fraction of component 2.

$\beta = 1 - \mu_2/\mu_1$

	$X_2^\circ = 0.1$					$X_2^\circ = 0.4$			
γ ↓	$\beta \rightarrow 0.1$	0.4	0.7	0.9	γ ↓	$\beta \rightarrow 0.1$	0.4	0.7	0.9
0.1	1.27	2.65	6.9	22	0.1	1.27	3.24	29	$>10^5$
.2	1.18	1.98	3.59	6.2	.2	1.18	2.24	7.7	560
.3	1.13	1.66	2.54	3.68	.3	1.13	1.82	4.06	23
.4	1.10	1.47	2.03	2.62	.4	1.10	1.57	2.77	6.5
.5	1.07	1.34	1.70	2.05	.5	1.08	1.40	2.09	3.43
.6	1.05	1.23	1.48	1.68	.6	1.06	1.28	1.69	2.27
.7	1.04	1.16	1.32	1.43	.7	1.04	1.19	1.43	1.73
.8	1.02	1.09	1.18	1.26	.8	1.02	1.12	1.26	1.40
.9	1.01	1.04	1.08	1.12	.9	1.01	1.05	1.11	1.17
	$X_2^\circ = 0.7$					$X_2^\circ = 0.9$			
γ ↓	$\beta \rightarrow 0.1$	0.4	0.7	0.9	γ ↓	$\beta \rightarrow 0.1$	0.4	0.7	0.9
0.1	1.28	3.87	93	$>10^7$	0.1	1.28	4.50	170	$>10^8$
.2	1.19	2.54	20	$>10^4$	.2	1.19	2.81	34	$>10^5$
.3	1.13	2.01	8.1	$>10^3$	.3	1.14	2.15	13.1	$>10^4$
.4	1.10	1.70	4.55	160	.4	1.11	1.79	6.7	$>10^3$
.5	1.08	1.49	3.00	24	.5	1.08	1.55	4.04	200
.6	1.06	1.34	2.19	6.9	.6	1.06	1.38	2.74	39
.7	1.04	1.23	1.69	3.07	.7	1.04	1.26	2.03	10.5
.8	1.02	1.14	1.37	1.88	.8	1.02	1.15	1.54	3.74
.9	1.01	1.07	1.16	1.32	.9	1.01	1.07	1.23	1.73

Experimental

The two component systems studied were prepared from Armour and Company crystallized bovine plasma albumin and bovine plasma fraction II. The latter was refractionated by electrophoresis–convection in *p*H 5.8 phosphate buffer of ionic strength 0.1 to give a top fraction of bovine γ-globulin of mobility -1.81×10^{-5} cm. sec.$^{-1}$ volt^{-1} in *p*H 8.6 ionic strength 0.1 barbital buffer. The γ-globulin thus obtained was concentrated by pervaporation and mixed with a solution of the albumin to make up mixtures of the composition ratios given in Table II. The solutions were dialyzed 24 hours against *p*H 8.10 phosphate buffer of ionic strength 0.1 and then fractionated by electrophoresis–convection in this buffer under the conditions of Table II. All the fractionations were carried out in a refrigerated room maintained at 3.5°. Each run was discontinued after a suitable period of time and the contents of the top reservoir removed and analyzed.

The total protein content of the solutions was determined by the Nessler reagent method of Koch and McMeekin.[5] The fraction of protein remaining in the top reservoir was corrected for osmosis by multiplication by the ratio of the total final solution volume to the initial volume. The relative amounts of each component were determined from measurements of the areas in descending boundary electrophoretic patterns obtained from a Tiselius apparatus by the schlieren scanning method. The electrophoresis runs for this purpose were all made in *p*H 8.6 barbital buffer of ionic strength 0.1. The original mixtures and top fractions were run at several dilutions each and the component and total areas exclusive of the ϵ-boundary were measured with a planimeter on projected tracings of the photographic patterns. The precision of these results was not sufficient to permit reliable extrapolations of the area ratios to zero protein concentration at constant ionic strength to obtain the "true" component ratios as recommended by Longsworth.[6] However, at low concentrations within the limits of experimental error the area ratios appear to change but slowly with protein concentration in agreement with the calculations of Longsworth[6] for a similar system. Consequently the component ratios were based upon averages taken of the values obtained at total protein concentrations less than 1.4 g. per 100 ml. In the case of the original mixtures, which were prepared from measured volumes of solutions of known protein content, the electrophoretic analyses resulting from this procedure agreed with those calculated from the volumes to within 0.02 g. per 100 ml. for each component.

The electrophoretic mobilities were also measured by the moving boundary technique and were -1.61×10^{-5} and -8.28×10^{-5} cm. sec.$^{-1}$ volt^{-1} for γ-globulin and albumin, respectively, in the *p*H 8.10 phosphate buffer as corrected to 3.5°.

TABLE II

FRACTIONATION OF BOVINE γ-GLOBULIN AND ALBUMIN BY ELECTROPHORESIS–CONVECTION[a]

Run	C°[b] g./100 ml.	X_2°	E, v./cm.	γ	X_2^c	f_2	$X_{2\text{calcd.}}$	$f_{2\text{calcd.}}$	E^*/E
1	2.40	0.40	0.100	0.660	0.51	1.56	0.53	1.71	0.44
2	2.37	.40	1.64	.238	.94	23.5	.90	13.3	.22
3	2.20	.11	1.64	.081	.75	24.3	.68	16.4	..
4	1.71	.40	1.64	.404	.79	5.65	.72	3.85	.24
5	1.71	.40	1.64	.178	.96	36	.96	35	.25

[a] Subscript 2 refers to the γ-globulin component. [b] C° is the initial total protein concentration. [c] X_2 is the weight fraction of γ-globulin in the protein remaining in the top reservoir at the completion of the run.

The buffer circulation system for the electrophoresis–convection cell is such that 5% of the electric current leaks through the external buffer and is not effective in transporting protein.[2] Hence, the nominal field strengths reported in Table II are 95% of those calculated from the measured currents, channel area and specific resistance of the buffer.

Results

In Table II along with the experimentally observed weight fractions of γ-globulin remaining in the protein of the top reservoir at the end of each run and the separation factors calculated from them are presented the separation factors and final weight fractions predicted theoretically from Eq. (1) based upon the mobilities, initial weight fractions of γ-globulin, X_2°, and final total protein concentrations,

(5) F. C. Koch and T. L. McMeekin, THIS JOURNAL, **46**, 2066 (1924).

(6) L. G. Longsworth, *J. Phys. & Colloid Chem.*, **51**, 171 (1947).

γ. The agreement between experimental and theoretical values is seen to be good.

The derivation of Eq. (1) assumes electrophoretic homogeneity of the two proteins. However, the γ-globulin did not meet this requirement and in the experiments which were carried out to very small final total protein concentrations some further fractionation of the γ-globulin was observed. In run 3 for example the mean mobility of the residual γ-globulin in the top reservoir was found to have changed from -1.81×10^{-5} to -1.61×10^{-5} cm. sec.$^{-1}$ volt^{-1} in barbital buffer with no apparent change in mobility of the albumin. This behavior largely explains the fact that the experimental separation factors particularly in the longer runs tend to be larger than the theoretical ones since the fractionation improves as the difference in mobilities of the two components increases.

Although no effort was made to vary the ratio of mobilities of these two proteins, a case involving a different ratio is afforded by a program of experiments designed to fractionate insulin by electrophoresis–convection which has been carried out in these laboratories. In a typical run: $C^0 = 1.63$, $E = 1.39$, $X_2^\circ = 0.19$, $\gamma = 0.10$, $X_2 = 0.30$, $f_2 = 1.71$. The theoretical values of X_2 and f_2 are 0.31 and 1.90, respectively. Thus satisfactory agreement is shown between experimental and theoretical values of the final ratio of component concentrations in the top reservoir. The mean mobilities of the two electrophoretic components of insulin are -6.37×10^{-5} and -4.98×10^{-5} in the *p*H 8.5 ionic strength 0.1 borate buffer used in the fractionations.

In the experimental study of single protein systems it was found that the time required to transport a specified fraction of protein from the top to the bottom reservoir is adequately represented by the theory provided the nominal field strength, E, is replaced by an empirical effective field strength E^*, which must be determined experimentally for each system and each set of operating conditions. The ratio E^*/E varies from about 1.3 to 0.24 being smaller for large values of E and depending upon the system being investigated. The calculations of the time of transport as a function of the fraction of protein transported for the two component system of runs 1, 2, 4 and 5 have been carried out and the results of comparison of experiment with theory are presented in Table II by means of the ratio E^*/E. This ratio can be seen to be of the same magnitude and to show the same type of dependence upon E as in the single component study. In run 5 the total protein concentration of the top reservoir was followed as a function of time by sampling at suitable intervals as in the single component investigation.[2] Within experimental error the points fall on the theoretical curve calculated with a field strength of 0.42 volt per cm. In runs 1, 2 and 4 only the final concentrations of the top reservoir were measured and the values of E^*/E are based upon these points alone.

A number of simplifying assumptions underlying the derivation of the theory of electrophoresis–convection are not exactly realized in practice and would be expected to contribute to quantitative disagreement with experiment. Most important of these is the assumption that $\mu_{max} Ea/D_{min} << 1$ where a is the distance between membranes, usually about 0.1 cm., and D_{min} is the smallest of the protein component diffusion constants. Although this requirement is never fulfilled under practical operating conditions such as those of runs 2–5, the results of the theory apparently can be satisfactorily extended to these conditions by the inclusion of the empirical parameter E^*/E. Another assumption is that the electric field strength is everywhere constant and equal to that calculated from the specific resistance of the buffer. The actual electric field in the channel is certainly lower than this because of chemical potential gradients of the ion constituents in the channel and field jumps at the membranes where the protein ion constituents disappear. Also retarding the transport is the flow of solvent across the channel opposing the motion of the protein. This flow arises from an osmotic influx of solvent at the membrane at which the protein molecules accumulate and an efflux at the opposite depleted side.

The expression for the separation factors, Eq. (1), agrees satisfactorily with experiment over the entire range of variables studied. Thus, by means of Eq. (1) or Table I a reliable prediction of the degree of fractionation to be expected from a proposed fractionation may be made. Furthermore, the theory can be used to calculate the time required to transport a specified fraction of protein from top to bottom reservoir in two component systems provided the value of E^*/E can be estimated or is known from a pilot experiment under similar conditions.

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